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Putrescine Is Involved in the Vitamin D Action in Chick Intestine

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We have reported that a single injection of 1α,25-dihydroxyvitamin D_3 into vitamin D–deficient chicks produces a marked increase of putrescine accumulation in the duodenum from two different sources, ornithine and spermidine. In the present study, the effects of putrescine depletion and its supplementation on duodenal villus length and calcium absorption were examined in newborn and 5-week-old chicks. Administering either α-difluoromethylornithine, a specific inhibitor of ornithine decarboxylase, or N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine, a specific inhibitor of polyamine oxidase, to newborn chicks significantly decreased the duodenal content of putrescine and calcium transport activity. The putrescine depletion also induced shortening of the duodenal villus length. The inhibition of calcium absorption and villus length in the putrescine-depleted chicks was almost completely restored by administering putrescine to the birds. The effect of the putrescine depletion and its supplementation on the duodenal villus length and the calcium absorption was reproduced in 5-week-old vitamin D–deficient chicks given vitamin D_3 or 1α,25-dihydroxyvitamin D_3. These results clearly indicate that putrescine is somehow involved in the vitamin D action in maintaining the morphological and functional development of the intestinal villus mucosa.

Eukaryotic cells contain polyamines: spermidine, spermine, and their precursor, putrescine (1,2). Although the exact functions of the polyamines remain to be determined, it is established that their cellular concentrations are highly regulated and that they play an essential role in regulating cell growth and differentiation. Ornithine decarboxylase (ODC; E.C. 4.1.1.17) catalyzes the formation of putrescine from ornithine, a rate-limiting step in polyamine biosynthesis. Recently, several researchers have suggested the possibility that there is a conversion of the higher polyamines (spermidine and spermine) back into putrescine (3–6). The enzymatic reaction of such a reversal pathway has now been established (4,6,7). It involves two enzymes acting sequentially: spermidine/spermine N^1-acetyltransferase (SAT; E.C. 2.3.1.57) and polyamine oxidase (E.C. 1.5.3.3). Thus, putrescine is generated from both ornithine and spermidine/spermine.

Administering vitamin D_3 to rachitic animals causes a 2.5- to 3.5-fold increase in the rate of intestinal calcium transport activity after a time lag of 24–48 hours (8). This time lag is explained at least in part as the time required for the metabolic conversion of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the biologically active metabolite of the vitamin (9–11).

In previous research (12–14) we showed that the duodenal ODC and SAT activities increased markedly with a concomitant increase in the duodenal content of putrescine after a single injection of 1α,25$(OH)_2D_3$ into vitamin D–deficient chicks. The amount of the increase of duodenal putrescine after 1α,25$(OH)_2D_3$ injection into vitamin D–deficient chicks coincided quantitatively with the amount of N^1-acetylspermidine synthesized from spermidine after injection of the vitamin into the same vitamin D–deficient chicks pretreated with N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), a specific inhibitor of poly-

Abbreviations used in this paper: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; ALP, alkaline phosphatase; DFMO, α-difluoromethylornithine; MDL 72527, N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine; ODC, ornithine decarboxylase; SAT, spermine N^1-acetyltransferase.

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amine oxidase (15). This indicates that the increase in the putrescine content after $1\alpha,25(OH)_2D_3$ administration is mainly due to the conversion of spermidine into putrescine.

In 1972, Spielvogel et al. (16) reported that the daily administration of vitamin D_3 to vitamin D–deficient chicks for 4 days resulted in a considerable (30%) increase in the villus length. Birge and Alpers (17) also reported that administration of vitamin D_3 to rachitic rats increased the duodenal synthesis of DNA and elongated the villus. These results led us to examine the relationship between polyamine metabolism and epithelial cell growth and differentiation in intestine.

In the present study, the in vivo effect of putrescine depletion and its supplementation on the duodenal villus length and calcium absorption were examined in chicks. Simultaneous administration of α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, and MDL 72527 greatly shortened the duodenal villus length and inhibited calcium absorption. These were almost completely restored by adding putrescine. It is therefore reported that putrescine is somehow involved in the vitamin D action in maintaining the morphological and functional development of the intestinal villus mucosa.

Materials and Methods

Animals

For studies of polyamine metabolism before and after hatching of chick embryos, fertilized White Leghorn eggs were obtained from a local distributor and incubated at 37.5°C in a humidified egg incubator. After hatching, the birds were fed a commercial diet and water ad libitum. For studies of polyamine depletion and its supplementation, 1-day-old White Leghorn cockerels were purchased from the Goto Poultry Farm (Gifu, Japan) and raised for 5 weeks on a synthetic vitamin D–deficient diet containing 1.0% calcium and 0.45% phosphorus (18). For the last 2 weeks, vitamin D_3 was administered orally in 100 μL of cottonseed oil every day. Some birds received IV 0.625 μg of $1\alpha,25(OH)_2D_3$ dissolved in 50 μL of ethanol.

Chemicals

Putrescine dihydrochloride and spermidine trihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin D_3 was obtained from Wako Pure Chemicals (Osaka, Japan). [acetyl-1-^{14}C]Acetylcoenzyme A (1.67 GBq/mmol) and D,L-[1-^{14}C]ornithine hydrochloride (1.9 GBq/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]Thymidine (4.77 TBq/mmol) was obtained from Amersham International plc (Buckinghamshire, England). α-Difluoromethylornithine (MDL 71782) was kindly donated by Dr. P. P. McCann of Merrell Dow Pharmaceuticals Inc. (Cincinnati, OH). N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine was synthesized in our laboratory according to the method of Bey et al. (19). The nuclear magnetic resonance spectrum of the product fully corroborated the assigned structure, and its melting point (241–242°C) coincided with that reported by Bey et al. (19), indicating that the synthesized product was pure. All other chemicals were of analytical grade.

Enzyme Assays for Polyamine Metabolism

The activities of ODC and SAT were measured as described previously (13). In short, birds and chick embryos were killed by decapitation, and the duodenum was quickly removed and washed thoroughly with ice-cold 0.15 mol/L NaCl. The intestinal tissues were placed on ice, and the mucosal layer was scraped from the underlying muscle layers and homogenized with 9 volumes of 50 mmol/L Tris/HCl (pH 7.5). The homogenate was centrifuged at 30,000*g* for 30 minutes, and the resulting supernatant was used as an enzyme solution. Activity of ODC was measured by incubating 0.4 mL of a 30,000*g* supernatant with 0.2 μCi of D,L-[1-^{14}C]ornithine/HCl in a medium containing 50 mmol/L Tris/HCl (pH 7.5), 5 mmol/L dithiothreitol, 0.2 mmol/L pyridoxal phosphate, and 0.1 mmol/L ornithine in a total volume of 0.5 mL. After incubation for 30 minutes at 37°C, the reaction was terminated by adding 0.1 mL of 2 mol/L citric acid. The radioactivity in the evolved $^{14}CO_2$ was counted with a liquid scintillation counter. The assay system for SAT contained 300 nmol of spermidine, 10 μmol of Tris/HCl (pH 7.8), and 0.8 nmol (40 nCi) of [acetyl-1-^{14}C]acetylcoenzyme A in a total volume of 0.1 mL. After incubation for 5 minutes at 25°C, the reaction was terminated by adding 20 μL of 1 mol/L NH_2OH/HCl and heating for 3 minutes in a boiling water bath. Forty microliters of the reaction mixture was then applied to a Whatmann P-81 phosphocellulose filter disk, which was thoroughly washed with distilled water then dried. The radioactivity on the filter disk was counted as described above.

Analysis of Polyamines

The polyamines were analyzed on a Hitachi automated amino acid analyzer (model 835; Hitachi, Tokyo, Japan) by a modification of the method of Tabor et al. (20) as described in a previous paper (13). Briefly, tissues were thoroughly washed with 0.15 mol/L NaCl and homogenized with 9 volumes of perchloric acid. After the precipitates were removed by centrifugation, samples of the supernatant were used for polyamine analysis as described in the previous paper (13).

Duodenal Calcium Absorption

The duodenal calcium absorption activity was determined by the in situ duodenal loop method of Omdahl et al. (18) as described in a previous paper (12). In short, the duodenum was exposed via an abdominal incision, and

both ends of a 4 cm section were ligated. A test solution containing 20 mmol/L $CaCl_2$, 0.5 μCi/mL $^{45}CaCl_2$, 120 mmol/L NaCl, 4.9 mmol/L KCl, and 10 mmol/L HEPES (pH 7.5), was injected with a calibrated syringe (100 μL for 4-day-old chicks and 200 μL for 5-week-old chicks). Thirty minutes after the injection, the chicks were killed and the loops were removed, dried, and dry-ashed in porcelain crucibles. The radioactivity in the residue was counted in an ACS-II aqueous counting scintillant (Amersham, Arlington Heights, IL). The results were calculated by the formula

$$\%\ ^{45}Ca_{abs} = (1 - {}^{45}Ca_P/{}^{45}Ca_A) \times 100,$$

where $^{45}Ca_R$ is the amount of ^{45}Ca remaining in the loop after the in situ incubation and $^{45}Ca_A$ is the amount of ^{45}Ca initially added to the loop preparation.

Miscellaneous

[^{3}H]Thymidine in 0.15 mol/L NaCl was injected IP at a dose of 10 μCi/100 g body wt. One hour after the [^{3}H]thymidine injection, animals were killed, and the radioactivity in the trichloroacetic acid–insoluble fraction was counted. The DNA content was determined by the method of Burton (21). The activity of alkaline phosphatase (ALP; E.C. 3.1.3.1) was measured at pH 10.5 by the Kind–King method with *p*-nitrophenylphosphate as substrate. Protein levels were determined by the method of Smith et al. (22) using bovine serum albumin as a standard. Student's *t* test was used to determine the statistical significance of the differences between the experimental and control groups.

Results

Changes in Duodenal Ornithine Decarboxylase, Spermidine N^1-Acetyltransferase, and Alkaline Phosphatase Activities in Chicks Before and After Hatching

The time courses of change in the activities of ODC, SAT, and ALP before and after hatching is shown in Figure 1. The activities of the three enzymes were detectable 4 days before hatching. In agreement with the report of Moruzzi et al. (23), the ODC activity increased before hatching, attained a maximum at hatching, and decreased thereafter (Figure 1*A*). The SAT activity, in contrast, increased progressively after hatching (Figure 1*A*). Alkaline phosphatase, a marker enzyme of the maturation of intestinal epithelial cells, increased just before hatching, and the elevated level was maintained after hatching (Figure 1*B*).

Figure 2 shows the time courses of change in the

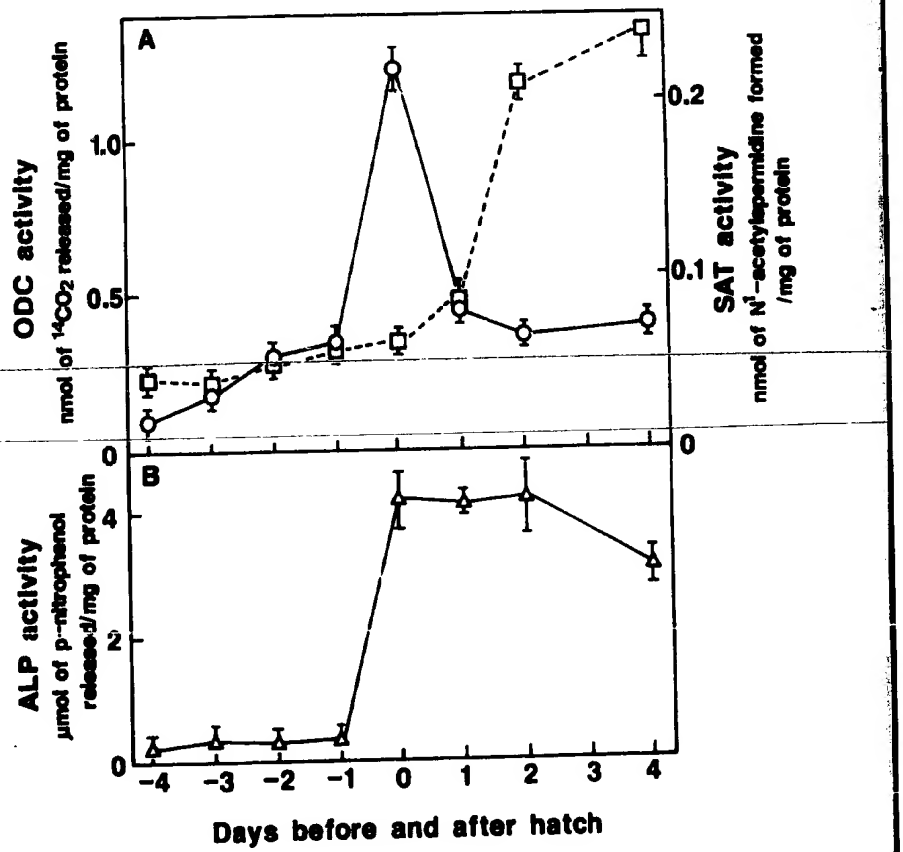

Figure 1. Time courses of change in the ODC, SAT, and ALP activities of chick duodenal mucosa before and after hatching. Results are means ± SE of four to five determinations. The number of birds used in each determination was 4 on day −4, 3 on day −3, 2 on days −2 and −1, and 1 on days 0–4.

A. duodenal ODC (○—○) and SAT (□···□).

B. ALP (△—△).

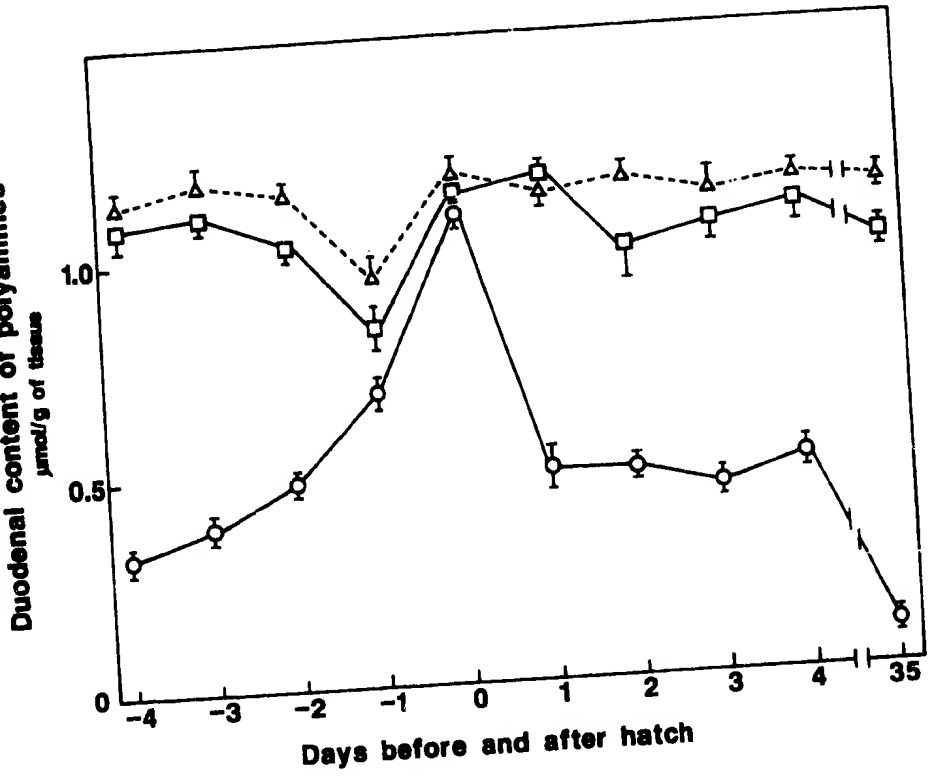

Figure 2. Time courses of change in the duodenal content of polyamines before and after hatching. The duodenal contents of putrescine (○—○), spermidine (□—□), and spermine (△---△) at each time point are means ± SE of four to five determinations. The number of birds used in each determination was 4 on day −4, 3 on day −3, 2 on days −2 and −1, and 1 on days 0–4.

duodenal content of the polyamines before and after hatching. The duodenal contents of spermidine and spermine gradually decreased toward hatching during embryonic development, but they returned to the elevated levels after hatching. These two time courses were almost the same as those in chicks raised for 4–5 weeks on a vitamin D–deficient diet (12–14). In contrast, the duodenal content of putrescine before and after hatching was 5–10 times higher than that of the 5-week-old vitamin D–deficient birds. The putrescine content increased toward hatching and then decreased, which was in accord with the change in the ODC activity (Figures 1A and 2).

In Vivo Effects of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on the Duodenal Ornithine Decarboxylase and Spermidine N¹-Acetyltransferase Activities

Figure 3 shows the in vivo effects of DFMO and MDL 72527 on duodenal ODC and SAT activities. Administering DFMO suppressed the duodenal ODC activity almost completely, but MDL 72527 did not affect it. Duodenal SAT activity was also inhibited slightly by the administration of DFMO ($P < 0.05$), but it was not changed by the treatment with MDL 72527 alone or with MDL 72527 and DFMO.

In Vivo Effects of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on the Duodenal Content of Polyamines

Table 1 shows the effect of DFMO and MDL 72527 on the duodenal content of polyamines. Administering DFMO or MDL 72527 decreased the duodenal content of putrescine to 57% and 47%, respectively, of the levels in the control birds. Simultaneous treatment with DFMO and MDL 72527 decreased the putrescine content to less than 10% of that measured in the control. The duodenal content of spermidine was decreased by the treatment with DFMO alone or DFMO plus MDL 72527, but it remained unchanged by the treatment with MDL 72527 alone. Administering putrescine to those chicks with low polyamine levels restored the duodenal content of putrescine and spermidine almost completely. The duodenal levels of putrescine and spermidine significantly increased as early as 12 hours after the putrescine administration in birds treated with DFMO or DFMO plus MDL 72527. Administering MDL 72527 alone did not affect the duodenal content of spermidine, though it significantly decreased the putrescine content. Treatment with MDL 72527 caused a marked accumulation of N^1-acetylspermidine and N^1-acetylspermine in the duodenum of birds treated with and without DFMO. The duodenal content of spermine

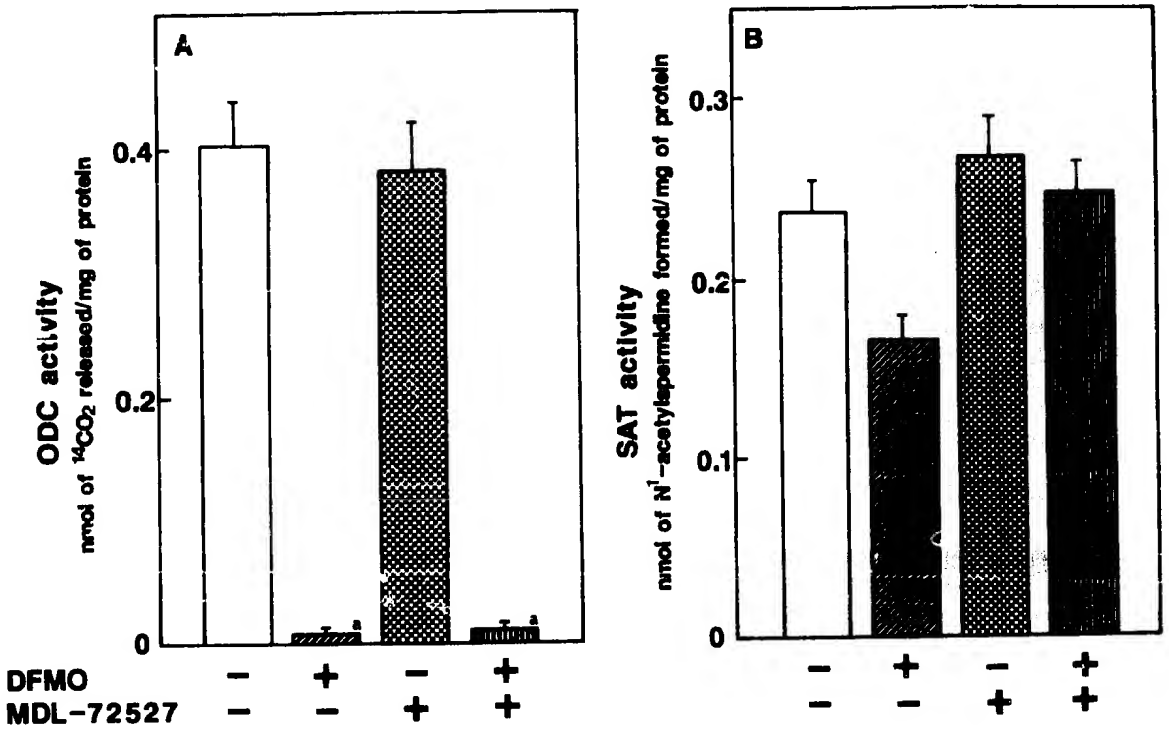

Figure 3. Effect of daily administration of DFMO and MDL 72527 on duodenal ODC and SAT activities. Birds received DFMO as a 2% solution in drinking water for 4 days after a single oral administration of DFMO (20 mg/100 g body wt) at the time of hatching. MDL 72527 was given orally at a dose of 2 mg/100 g body wt every 12 hours. Results are means ± SE of four birds. [a]Significantly different from the birds without any treatment ($P < 0.01$).

was not changed by any treatment. Concomitantly with the decrease in duodenal spermidine levels, treatment for 4 days with DFMO or DFMO plus MDL 72527 also reduced the duodenal fresh weight (control, 433.0 ± 4.5 mg/4 cm; DFMO, 339.6 ± 6.7 mg/4 cm; DFMO plus MDL 72527, 353.1 ± 2.0 mg/4 cm). The duodenal weight loss was restored almost completely by adding putrescine for the last 3 days or even 12 hours after a single injection of putrescine [DFMO given birds treated with putrescine for 3 days

Table 1. Effects of Daily Administration of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on Duodenal Content of Polyamines

Treatment	Putrescine (nmol/g tissue)	Spermidine (nmol/g tissue)	Spermine (nmol/g tissue)	N^1-Acetylspermidine (nmol/g tissue)	N^1-Acetylspermine (nmol/g tissue)
Control	508.3 ± 24.1	1061.3 ± 24.2	1446.3 ± 131.8	<2.0	<2.0
DFMO	290.3 ± 36.4[a]	629.4 ± 54.9[a]	1351.9 ± 44.0	17.8 ± 1.3	<2.0
MDL-72527	237.7 ± 34.7[a]	1037.9 ± 80.1	1430.1 ± 113.6	107.2 ± 6.3	52.0 ± 6.4
DFMO + MDL-72527	45.9 ± 8.6[a]	488.3 ± 47.1[a]	1588.0 ± 63.9	98.8 ± 8.6	36.4 ± 4.3
DFMO + putrescine (3 days)	445.5 ± 81.8	1018.8 ± 29.7	1424.1 ± 105.1	<2.0	<2.0
MDL-72527 + putrescine (3 days)	425.3 ± 12.4	1122.4 ± 63.2	1437.9 ± 76.3	87.7 ± 6.2	43.3 ± 7.9
DFMO + MDL-72527 + putrescine (3 days)	416.6 ± 43.7	1031.5 ± 52.8	1517.6 ± 55.5	47.4 ± 4.2	22.0 ± 1.0
DFMO + MDL-72527 + putrescine (12 h)	195.7 ± 21.9[a]	890.3 ± 55.1	1386.7 ± 32.5	46.4 ± 8.8	<2.0
DFMO + putrescine (12 h)	578.0 ± 82.4	952.0 ± 117.9	1545.4 ± 64.9	11.1 ± 1.1	<2.0

NOTE. Chicks received DFMO as a 2% solution in drinking water for 4 days after a single oral administration of DFMO (20 mg/100 g body wt) at the time of hatching. MDL 72527 dissolved in 0.1 mL of 0.15 mol/L NaCl was given orally every 12 hours. Putrescine was given orally as a 0.02% solution in drinking water for the last 3 days or as a single administration at a dosage of 20 mg/100 g body wt 12 hours before death. All birds were killed on day 4. The results are means ± SE of four birds.

[a]Significantly different from the control group ($P < 0.01$).

(409.0 ± 16.4 mg/4 cm) and 12 hours (392.8 ± 9.5 mg/4 cm); DFMO plus MDL 72527 given birds treated with putrescine for 3 days (497.1 ± 60.4 mg/4 cm) and 12 hours (370.7 ± 20.8 mg/4 cm)]. The duodenal weight was not changed appreciably by the treatment with MDL 72527 alone (413.2 ± 8.1 mg/4 cm).

In Vivo Effects of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on Duodenal Calcium Absorption

Figure 4 shows the in vivo effects of DFMO and MDL 72527 on duodenal calcium absorption and ALP activity. Administering either DFMO or MDL 72527 significantly decreased duodenal calcium absorption (Figure 4*A*). Simultaneous administration of the two inhibitors decreased it more effectively than either administration alone. Duodenal calcium transport activity returned to its control level 3 days or even 12 hours after putrescine administration. In contrast, duodenal ALP activity was not affected appreciably by administering either DFMO, MDL 72527, or both (Figure 4*B*).

Administering DFMO and MDL 72527 caused a significant decrease in the intestinal villus length. A typical example of this effect is shown in Figure 5. The villus length of a bird given DFMO and MDL 72527 (Figure 5*B*) was considerably shorter than that of a control bird (Figure 5*A*), and it elongated nearly to the control level as early as 12 hours after putrescine administration (Figure 5*C*). Table 2 summarizes the effects of the administration of DFMO and MDL 72527 on duodenal villus length. The average villus length in the birds given DFMO, MDL 72527, or DFMO plus MDL 72527 was 67.7%, 74.2%, and 65.4% of the control value, respectively. Administering putrescine to the birds given the two inhibitors increased the villus length considerably. The average villus length of the birds given putrescine returned to 92.5% and 105.5% of the values found in control birds 12 hours and 3 days, respectively, after the daily administration of putrescine.

Table 3 shows the effect of in vivo administration of

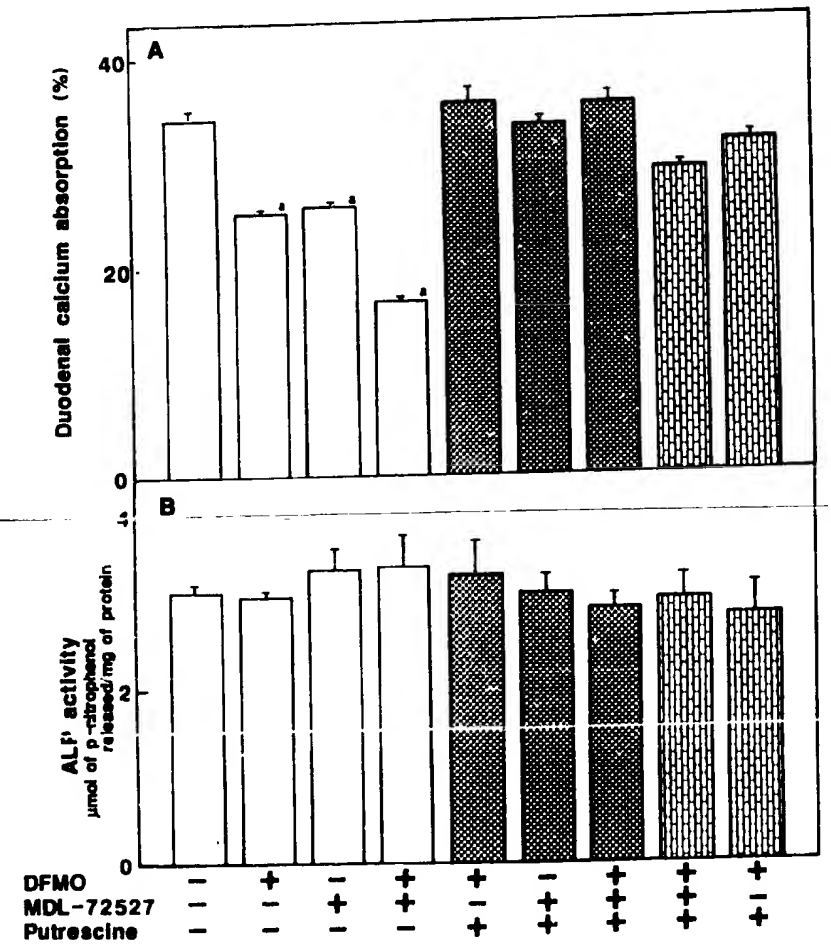

Figure 4. Effect of daily administration of DFMO and MDL 72527 on duodenal calcium absorption (*A*) and ALP activity (*B*). Birds received DFMO as a 2% solution in drinking water for 4 days after a single oral administration of DFMO (20 mg/100 g body wt) at the time of hatching. MDL 72527 was given orally at a dose of 2 mg/100 g body wt every 12 hours. ▨, Birds given putrescine as a 0.02% solution in drinking water for the last 3 days; ▩, birds given 20 mg putrescine/100 g body wt orally for 12 hours before death; □, birds not receiving putrescine. Results are means ± SE of four birds. *Significantly different from the birds without any treatment ($P < 0.01$).

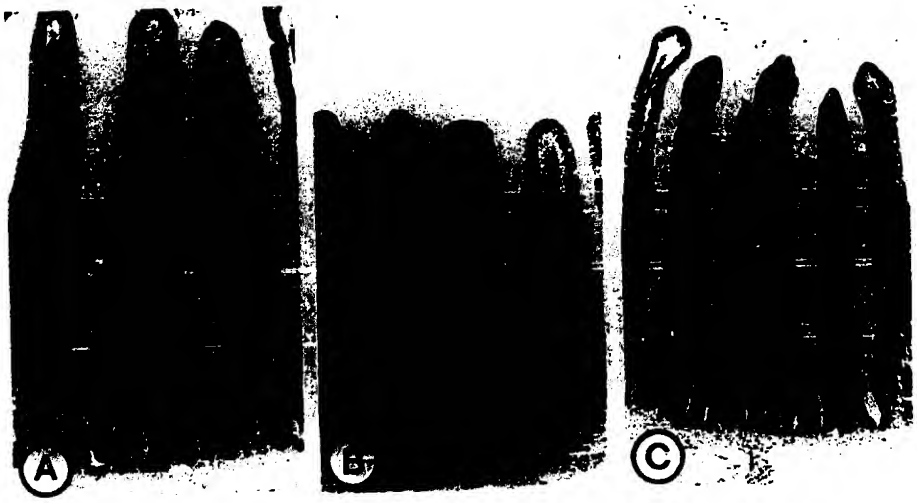

Figure 5. Longitudinal sections of the duodenal tissues of 4-day-old chicks (original magnification ×70).

A. Control bird.

B. A bird that received DFMO plus MDL 72527.

C. A bird that received DFMO and MDL 72527 and also putrescine (20 mg putrescine/100 g body wt) for the last 12 hours.

DFMO and MDL 72527 on the synthesis of duodenal DNA. Neither DFMO nor MDL 72527 decreased the synthesis of duodenal DNA. The simultaneous administration of the two inhibitors had no effect. Administering putrescine for the last 3 days to the birds given the two inhibitors did not affect the synthesis of duodenal DNA.

Effects of the Daily Administration of α-Difluoromethylornithine, N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine, and Vitamin D_3 on Intestinal Calcium Absorption in Vitamin D–Deficient Chicks

Figure 6 shows the effect of daily administration of DFMO, MDL 72527, and vitamin D_3 on intestinal calcium absorption in vitamin D–deficient chicks. The daily administration of 0.625 μg of vitamin D_3 for 2 weeks to chicks fed a vitamin D–deficient diet for 3 weeks significantly increased intestinal calcium transport activity ($P < 0.01$). Daily administration of either DFMO or MDL 72527 for the last 2 weeks before death significantly decreased the rate of intestinal calcium absorption induced by vitamin D_3. The decrease induced by DFMO was restored completely by adding putrescine. The daily administration of DFMO completely inhibited ODC activity in all cases, though it did not influence SAT activity (data not shown). Daily administration of MDL 72527 did not affect the activities of ODC or SAT (data not included). The mean villus length of vitamin D–deficient chicks was increased from 1738.4 ± 23.5 to 2015.2 ± 49.6 μm by the daily supplementation of 0.625 μg of vitamin D_3 for 2 weeks. The villus length of vitamin D_3–supplemented chicks was shortened by concomitantly administering either DFMO for 2 weeks to 1410.4 ± 30.6 μm ($P < 0.01$) or MDL 72527 for 2 weeks to 1473.1 ± 28.9 μm ($P < 0.01$). The shortened villus length was restored to the control level by administering putrescine (1953.2 ± 18.6 μm in the DFMO-treated birds and 1805.6 ± 20.2 μm in the MDL 72527–treated birds).

Table 4 compares the in vivo effects of DFMO, MDL 72527, putrescine, and spermidine on the 1α,-25$(OH)_2D_3$-induced duodenal calcium absorption. The duodenal calcium absorption was determined 12 hours after a single IV injection of 0.625 μg of 1α,25$(OH)_2D_3$ into 5-week-old vitamin D–deficient chicks. The vitamin increased calcium transport activity about fourfold. Prior administration of MDL 72527

Table 2. Effects of Daily Administration of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on Duodenal Villus Length

Treatment	Duodenal villus length (μm)
Control	923.6 ± 28.4 (100%)
DFMO	625.3 ± 3.2* (67.7%)
MDL-72527	685.5 ± 28.9* (74.2%)
DFMO + MDL-72527	603.7 ± 19.6* (65.4%)
DFMO + MDL-72527 + putrescine (3 days)	974.3 ± 26.8 (105.5%)
DFMO + MDL-72527 + putrescine (12 h)	852.5 ± 23.9 (92.3%)

NOTE. Chicks received DFMO as a 2% solution in drinking water for 4 days after a single oral administration of DFMO (20 mg/100 g body wt) at the time of hatching. MDL 72527 dissolved in 0.1 mL of 0.15 mol/L NaCl was given orally every 12 hours. Chicks were given putrescine as a 0.02% solution in drinking water for the last 3 days or as a single oral administration at a dosage of 20 mg/100 g body wt 12 hours before death. All birds were killed on day 4. Samples were always collected from the descending duodenal loop 3 cm from the distal end of the gizzard. Results are means ± SE of four birds. The values in parentheses are percentages of the control.

*Significantly different from control group ($P < 0.01$).

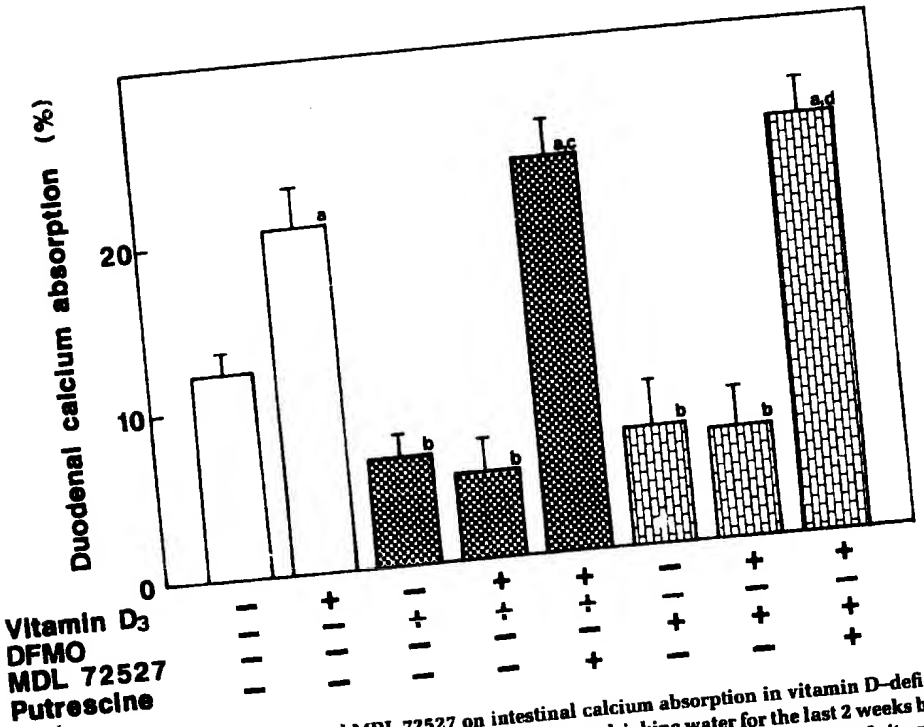

Figure 6. Effect of daily administration of DFMO and MDL 72527 on intestinal calcium absorption in vitamin D–deficient birds. Birds fed a vitamin D–deficient diet for 5 weeks were given DFMO (⊠) as a 2% solution in drinking water for the last 2 weeks before death. MDL 72527 (⊞) was given orally at a dose of 2 mg/100 g body wt every 12 hours. They were also given orally 0.625 μg of vitamin D_3 dissolved in 50 μL of cottonseed oil daily for the same 2-week feeding period. The control birds received cottonseed oil. Putrescine (20 mg/100 g body wt) was administered orally 12 hours before death. The results are means ± SE of five to eight birds. [a]Significantly different from the vitamin D–deficient control group ($P < 0.01$). [b]Significantly different from the vitamin D_3–treated control group ($P < 0.01$). [c]Significantly different from the group treated with vitamin D_3 and DFMO ($P < 0.01$). [d]Significantly different from the group treated with vitamin D_3 and MDL 72527 ($P < 0.01$).

(2 mg/100 g body wt 2 hours before the $1\alpha,25(OH)_2D_3$ injection) significantly decreased the duodenal rate of absorption of calcium induced by $1\alpha,25(OH)_2D_3$ ($P < 0.01$), though it affected neither ODC nor SAT activity (data not included). Daily administration of DFMO (2% solution in drinking water) also decreased

Table 3. Effects of Daily Administration of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on Synthesis of Duodenal DNA

Treatment	DNA synthesis (pmol [³H]thymidine incorporated/mg DNA)
Control	0.90 ± 0.03
DFMO	0.97 ± 0.05
MDL-72527	0.83 ± 0.09
DFMO + MDL-72527	0.74 ± 0.10
DFMO + MDL-72527 + putrescine (3 days)	0.88 ± 0.07

NOTE. Chicks received DFMO as a 2% solution in drinking water for 4 days after a single oral administration of DFMO (20 mg/100 g body wt) at the time of hatching. MDL 72527 dissolved in 0.1 mL of 0.15 mol/L NaCl was given orally every 12 hours. Chicks were given putrescine as a 0.02% solution in drinking water for the last 3 days. All birds were killed on day 4. Results are means ± SE of four birds.

Table 4. Effect of Daily Administration of α-Difluoromethylornithine and N¹,N⁴-Bis(2,3-butadienyl)-1,4-butanediamine on $1\alpha,25(OH)_2D_3$-Induced Duodenal Calcium Absorption

Treatment	Calcium absorption (%)
Control	10.8 ± 1.1 (100%)
$1\alpha,25(OH)_2D_3$	46.8 ± 4.1[a] (433%)
$1\alpha,25(OH)_2D_3$ + DFMO	32.6 ± 1.7[a] (302%)
$1\alpha,25(OH)_2D_3$ + MDL 72527	18.6 ± 3.3[b] (172%)
$1\alpha,25(OH)_2D_3$ + DFMO + putrescine	41.1 ± 1.8[a] (381%)
$1\alpha,25(OH)_2D_3$ + MDL 72527 + putrescine	39.4 ± 1.2[a] (365%)
$1\alpha,25(OH)_2D_3$ + DFMO + spermidine	9.7 ± 1.3[b] (90%)
$1\alpha,25(OH)_2D_3$ + MDL 72527 + spermidine	10.3 ± 1.8[b] (95%)

NOTE. Duodenal calcium absorption was measured 12 hours after a single IV injection of 0.625 μg of $1\alpha,25(OH)_2D_3$ into 5-week-old vitamin D–deficient chicks. DFMO was given orally as a 2% solution in drinking water for the last 2 weeks before death. MDL 72527 (2 mg/100 g body wt) was administered orally 2 hours before the $1\alpha,25(OH)_2D_3$ injection. Putrescine or spermidine was administered orally 6 hours before death at a dose of 20 mg/100 g body wt. Results are means ± SE of five to eight birds. Values in parentheses are percentages of the control.

[a]Significantly different from the vitamin D–deficient control group ($P < 0.01$).

[b]Significantly different from the $1\alpha,25(OH)_2D_3$-treated control group ($P < 0.01$).

the $1\alpha,25(OH)_2D_3$-induced calcium absorption, but the inhibitory effect of DFMO was smaller than that of MDL 72527. The decrease induced by either MDL 72527 or DFMO was restored completely by administering putrescine but not by spermidine (20 mg/100 g body wt for the last 6 hours).

Discussion

For the past few years, we have been examining the mechanism by which vitamin D regulates intestinal villus length from the standpoint of polyamine metabolism. The importance of polyamines in modulating cell growth and differentiation is well established (1–3). Luk et al. report that ODC plays an important role in the maturation of intestinal cells and their recovery from injury in rats (24). Dufour et al. (25) report that spermidine and spermine were involved in the maturation of rat intestinal epithelial cells. Lenzen et al. also report that spermidine stimulated calcium uptake by liver mitochondria (26). However, which polyamine has a critical role in certain tissues is still the subject of controversy. It may depend in part on the characteristic of polyamine metabolism in the respective tissues and cells. The present study shows that putrescine is somehow involved in the vitamin D action in maintaining the morphological and functional development of the intestinal villous mucosa, at least in chicks. In agreement with the results by Spielvogel et al. (16) and Birge et al. (17), the lengths of intestinal villi were shorter in the vitamin D–deficient chicks than in the vitamin D–supplemented birds. Administration of vitamin D into vitamin D–deficient birds elongated the villi to a normal range. In the present study, the administration of either DFMO or MDL 72527 significantly shortened the duodenal villi (Figure 5 and Table 2), and further administration of putrescine elongated them to within a normal range. This confirms that putrescine is involved in the mechanism of the action of $1\alpha,25(OH)_2D_3$ in regulating the intestinal villus length.

The specific role of putrescine has been reported in other cells. Erwin et al. (27) report that polyamine depletion inhibited in vitro differentiation of L6 myoblast cells and that this inhibition was almost completely restored by administering putrescine. Ginty et al. (28) report that in vitro treatment with putrescine significantly increased aphidicolin-sensitive DNA synthesis of serum-deprived intestinal epithelial cells (IEC-6). However, the elongation of duodenal villi by putrescine does not seem to be the result of an increase in DNA synthesis because it occurred as early as 12 hours after putrescine administration (Figure 5 and Table 2). In fact, the duodenal incorporation of [^{3}H]thymidine did not change after the administration of the inhibitors of polyamine metabolism or putrescine, separately or in combination (Table 3). Administering putrescine to polyamine-depleted birds elongated the duodenal villi without increasing DNA synthesis, resulting in longer, thinner villi. This may indicate that vitamin D elongates intestinal villi by stimulating differentiation of epithelial cells or the underlying muscular cells rather than by stimulating DNA synthesis of the crypt cells. The mechanism by which putrescine elongates duodenal villi is not known. Very recently, we found that transglutaminase is involved in the $1\alpha,25(OH)_2D_3$-induced fusion of mouse alveolar macrophages (29). Because this enzyme has been thought to be involved in the formation of crosslinks of various cytoskeletal proteins (30), it is likely that transglutaminase is involved in the differentiation of intestinal villus mucosa. Further studies are needed to clarify this possibility.

Putrescine not only elongated the duodenal villi but also enhanced duodenal calcium transport activity (Figures 4 and 6). The stimulatory effect of putrescine on calcium transport activity does not appear simply as a result of the maturation of intestinal villus mucosa because neither DFMO nor MDL 72527 affected duodenal ALP activity (Figure 4*B*). In agreement with our findings, Lucas et al. (31) report that polyamines are involved in the stimulation of calcium uptake by $1\alpha,25(OH)_2D_3$ into rat intestinal epithelial cells. They showed that 5 mmol/L DFMO abolished the increase of in vitro calcium uptake by $1\alpha,25(OH)_2D_3$. In contrast, Steeve et al. (32) report that administration of either DFMO or methylglyoxal bis(guanylhydrazone) for 3 days to vitamin D–deficient chicks did not inhibit the $1\alpha,25(OH)_2D_3$-induced duodenal calcium transport activity. Because ODC is not a rate-limiting enzyme for the duodenal synthesis of putrescine in 4- to 5-week-old chicks (14,15), the duodenal content of putrescine does not decrease appreciably after the daily administration of DFMO for 3 days. Furthermore, methylglyoxal bis(guanylhydrazone) has been reported to be a potent stimulator of SAT (33). Therefore, the administration of methylglyoxal bis(guanylhydrazone) may have stimulated the conversion of spermidine and spermine into putrescine. The mechanism by which putrescine stimulates intestinal calcium transport activity is not currently known. It is also likely that putrescine somehow elongates the duodenal villi, which in turn causes the increase in intestinal calcium transport activity. Further studies are needed to clarify this point.

In conclusion, putrescine is somehow involved in the vitamin D action in modulating the morphological and functional development of intestinal villus mucosa. The precise mechanisms of putrescine in stimu

lating duodenal calcium transport activity and the elongation of duodenal villi remain to be determined. We believe that the classical action of vitamin D in regulating intestinal calcium absorption can be explained at least in part by the recently discovered differentiation-inducing activity of $1\alpha,25(OH)_2D_3$.

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